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PARTIAL TENSION STREPTOCOCCI AND VACCINE PREPARATION¹

WADE W. OLIVER AND ORMAN C. PERKINS

From the Department of Bacteriology, Hoagland Laboratory, Long Island College Hospital, Brooklyn, New York

Although the respiration of bacteria has been especially reviewed by Kruse² and Meyer,³ yet by only a relatively few workers has attention been directed to the oxygen tension requirements of parasitic bacteria.

In this country, Rosenow's work on streptococci isolated from appendicitis, rheumatic joints, etc., and from glands draining the involved joints in arthritis deformans, is specially pertinent to the present discussion. He employed deep tubes of broth and agar. Of cultures from rheumatic joints, he says: "That the oxygen requirement is the chief factor to explain this difference in my results and the negative results of others is indicated also by the fact that the colonies never developed above 0.5 cm. from the top and never below 2 cm. from the bottom of the agar tubes. The largest number of colonies developed between 1.5 cm. from the top and 3.5 cm. from the bottom."⁴ Of cultures obtained from glands draining the lesions of arthritis deformans, he says: "All the streptococcal forms isolated have shown a marked preference for anaerobic conditions of growth in the primary culture."⁵ Further, he states: "It would seem as if in arthritis deformans the micro-organisms are taken up from the circulation by the endothelial cells which proliferate freely so that eventually the blood supply is reduced or cut off, in consequence of which there result areas of lowered oxygen tension, diminished nutrition and atrophy. Such conditions would favor the growth of organisms which on isolation are sensitive to oxygen."

As emphasized in a previous article,⁶ we have felt that the success-

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¹ Oliver: Preliminary Note, Long Island Med. Jour., 1918, 12, p. 132.

² Allg. Mikrobiologie, 1910.

³ Centralbl. f. Bakteriologie, I, O., 1909, 49, p. 305.

⁴ Jour. Infect. Dis., 1914, 14, p. 62.

⁵ Jour. Am. Med. Assn., 1914, 62, p. 1146.

⁶ Wherry and Oliver Jour. Infect. Dis., 1916, 19, p. 288.

ful cultivation of many known micro-organisms and of some of the unknown viruses of infectious diseases may be dependent more on the proper oxygen tension in the cultures than on the composition of the medium.

During the routine bacteriologic examinations at this laboratory within the past 12 months we have isolated from a variety of disease processes in the human body 32 strains of partial tension streptococci. Of these, 6 strains were isolated only by means of the partial tension method,⁶ aerobic and anaerobic cultures remaining sterile, and being discarded after from 5-15 days of incubation. The remaining 26 strains all yielded a decidedly more marked, and in certain cases luxuriant, growth in partial tension cultures than in aerobic and anaerobic cultures. In fact, the relative luxuriance of growth of many streptococci at partial tension is most striking.

TABLE 1
SOURCES AND OTHER DATA REGARDING THE STRAINS ISOLATED

Source of Streptococci	Number of Strains	Growth Only at Partial Tension	Vaccines Prepared
1. Sputum (asthma)	7	1	7
2. Pyorrhea	5	1	5
3. Mastoiditis	1
4. Throat	3
5. Furunculosis	3	..	3
6. Empyema	1	..	1
7. Uterus	8	2	..
8. Stool	1	..	1
9. Urine	1
10. "Cold Abscess"	2	2	..
Total	32	6	17

TECHNIC

1. *Sputum*.—In 7 cases suffering from marked asthma, streptococci were isolated in considerable numbers from the sputum. In 1 case, growth of streptococci was obtained only in the partial tension culture and in the remaining 6 cases growth was decidedly more marked under partial tension conditions than in aerobic or anaerobic cultures. Cultures were made as follows: Small masses of sputum were given 3 consecutive washings in sterile normal salt solution, after which they were streaked by means of a sterile glass spreader across human blood-agar (+1) plates. Partial tension conditions were supplied by incubating the plates at 37 C. in a sealed jar along with 3-5 plain agar (+1) slants inoculated with *B. subtilis* (Nowak).⁷ After 24-48 hours incubation, transplants from single colonies were made to slants of plain agar (+1) and dextrose agar (+1) and made partial tension by tightly connecting and incubating them at 37 C. with a plain agar (+1) slant of *B. subtilis*. By this method, the incubation of 4-6 tubes yielded sufficient growth within 24-48 hours to allow of the preparation of a vaccine. In no case was sufficient growth obtained on 3 days' incubation under aerobic and anaerobic conditions to admit of vaccine preparation.

⁷ Ann. de l'Inst. Pasteur, 1908, 22, p. 541.

2. *Pyorrhea*.—In 1 of the 5 cases a streptococcus was isolated only by means of partial tension cultures (plain agar (+1) and human blood agar (+1), no growth being obtained on these mediums under aerobic and anaerobic conditions. This case was one of severe pyorrhea for which rapid preparation of an autogenous vaccine was urgently requested. The partial tension method yielded a pure culture of a streptococcus which grew so well on plain agar (+1) that sufficient growth was obtained from 4 slants on 24 hours' incubation at 37 C. to allow of the preparation of a vaccine.

After preliminary swabbing of the gums with 95% alcohol, a small quantity of pus was expressed and obtained on a platinum loop. Plates were inoculated by the streak method. Isolation was effected by incubation at 37 C. of plain agar (+1) plates and human blood-agar (+1) plates made partial tension by the method of Nowak. After 24 hours' incubation, transplants of single colonies were made to partial tension slants of plain agar (+1) and dextrose agar (+1). In all 5 cases, vaccines were prepared from the growth obtained under diminished oxygen tension.

3. *Mastoiditis*.—In this case, pus obtained from the mastoid cells at operation was streaked in small quantities across plates of plain agar (+1) and human blood agar (+1). Both aerobic and anaerobic plates, when incubated at 37 C. for 24 hours, yielded growth. However, whereas in such cultures the growth consisted of isolated, tiny colonies, the growth obtained at partial tension, both on isolation and on subsequent transplants, was rather heavy and confluent.

Repeated transplants of the aerobic strain on plain agar every 24-48 hours results, after a number of transplants, in a more marked growth. The same applies to successive aerobic transplants made from the partial tension strain. This loss or modification of the oxygen tension requirement has frequently been noted. In reference to streptococci isolated from rheumatic joints, Rosenow⁸ states that "after cultivation from 1-8 months, the capacity to grow at a low temperature, the sensitiveness to oxygen, the excessive production of acid in dextrose broth, and the simultaneous affinity for joints, endocardium and myocardium are found to have largely or entirely disappeared."

We have very recently encountered an example of what we believe to be a complete and rather sudden modification of the oxygen requirement of a streptococcus within the body. Pus obtained from a cerebellar abscess in a young child showed, in direct smears, scattered gram +, tiny streptococci. Repeated smears were negative for acid-fast bacteria. Blood-agar plates were streaked with small quantities of the pus, and incubated at 37 C. aerobically and anaerobically and at partial tension. The aerobic and partial tension plates remained sterile, but anaerobically a considerable number of tiny, gray colonies of streptococci were obtained which failed to grow on aerobic and partial tension transplant. Eight days later, following operation,

⁸ Jour. Infect. Dis., 1914, 14, p. 66.

another specimen of pus was obtained. Direct smears showed enormous numbers of very small gram + streptococci. Aerobic cultures on human blood agar (+ 1), incubated for 24 hours at 37 C. revealed a few small colonies of streptococci and scattered colonies of staphylococci and a gram + bacillus. Six days later, a third specimen of pus when streaked on blood-agar (+ 1) plates yielded, on 24 hours' aerobic incubation at 37 C., a marked growth of streptococci. Macroscopically and microscopically, these streptococci were indistinguishable from the micro-organisms obtained in the first culture only under strict conditions of anaerobiosis. Although we realize that this is not an absolute criterion of the identity of the 2 strains, yet it would seem that the fact that in the second culture made 8 days after the original culture and following operation a few scattered colonies of streptococci were obtained on aerobic blood-agar plates points to a beginning change in the oxygen tension requirement of the previously sealed up micro-organism. Interpreted in this light, the marked aerobic growth obtained after another 6 days points to a most rapid adaptation of the streptococcus within the body to the altered oxygen environment obtained after operation and drainage.

4. *Throat*.—The 3 throat cultures were obtained from patients who were suffering from a moderately severe puerperal infection. These strains yielded a fairly marked growth aerobically on human blood-agar (+ 1) plates incubated at 37 C. and a very scant growth anaerobically. The growth on human blood agar (+ 1) at partial tension was somewhat greater than that obtained on aerobic incubation, but the difference in amount of growth was not so great as in the other cases reported in this article.

Swab cultures were taken from the tonsillar area of the throat and streak cultures made on human blood-agar (+ 1) plates, incubated at 37 C. Nowak's method was employed in making the cultures partial tension. The anaerobic cultures were made by sealing the plates in a jar containing pyrogalllic acid and potassium hydroxid.

5. *Furuncles*.—In the 3 cases of furunculosis, in which the partial tension method alone yielded sufficient growth to allow of the preparation of a vaccine, the surface of the boils was cleansed with bichlorid of mercury solution (1:1,000), followed by 95% alcohol. The lesion was then punctured with a sterile needle and the pus expressed. Human blood-agar plates streaked with a small quantity of pus were incubated at 37 C. The aerobic plates, on 24 hours' incubation at 37 C. yielded considerable numbers of colonies of *Staphylococcus pyogenes albus* and *aureus*, with a few, scattered, minute colonies of streptococci. The partial tension plates on 24 hours' incubation yielded larger colonies of the streptococcus in decidedly larger numbers. On transplant of single partial tension colonies to slants of dextrose agar (+ 1) incubated at 37 C., sealed along with slants of *B. subtilis*, sufficient growth was obtained from 5 tubes after 30 hours' incubation to allow of the preparation of vaccines.

6. *Empyema*.—A loopful of pus was streaked across 2 plates of human

blood agar (+1). A streptococcus was isolated in pure culture, the aerobic plate showing scattered colonies, whereas the plate at diminished oxygen tension showed a luxuriant growth of streptococci after 24 hours' incubation at 37 C. Transplants to plain agar slants, incubated at partial tension for 24 hours, yielded sufficient growth to allow of the preparation of a vaccine.

7. *Uterus*.—In 8 cases of uterine infection, a partial-tension streptococcus was isolated in pure culture. In 1 case growth was obtained only under conditions of diminished oxygen tension, and in the remaining 7 cases the marked growth at partial tension was in contrast to the rather delicate aerobic and anaerobic growth. The material for cultures was obtained by Dr. Beck by inserting a sterile glass tube, provided with a suction valve, into the uterus, and drawing into the tube the uterine secretion. The collecting tubes, wrapped in sterile towels, were immediately sent to the laboratory. The pus was removed from the tube by means of a small platinum loop and was streaked on human blood-agar (+1) plates with a sterile glass spreader.

Three of the patients, who suffered from a clinically similar, moderately severe uterine infection following childbirth and who were delivered with such extreme emphasis placed on asepsis as to practically exclude the possibility of outside infection, gave a history of influenza dating back from 1-4 months prior to delivery. From these patients a similar streptococcus was isolated from the uterus and from the throat.

One of the remaining 5 cases was that of a very severe chorea in a child. Material was obtained from the uterus in the manner previously described. An obligative partial tension streptococcus was isolated in pure culture on plates of Martin's ascitic agar (0.5),⁹ after 3 days' incubation at 37 C. Transplants of single colonies to human blood-agar (+1) slants incubated aerobically at 37 C. remained sterile, whereas transplants to slants of human blood agar (+1) incubated at 37 C. at partial tension yielded a rather luxuriant growth of streptococci.

8. *Stool*.—A loopful of stool was thoroughly suspended in 5 c.c. of sterile salt solution and 1 loopful of the suspension was spread on the surface of 2 human blood-agar (+1) plates. One plate was incubated aerobically at 37 C. and the other plate at 37 C. at partial tension. On 24 hours' incubation, the partial tension plate yielded a large number of colonies of streptococci, whereas the aerobic plate revealed only a few, scattered, tiny streptococcus colonies.

9. *Urine*.—One loopful of sediment, obtained by centrifuging 5 c.c. of urine at moderate speed for 20 minutes, was streaked across human blood-agar (+1) plates. The results were identical with those obtained in the case of the stool culture (cf. par. 8).

10. "*Cold Abscess*."—The report deals with 2 cases of "psoas abscess" developing in patients that, clinically, were suffering from tuberculosis.

A. Direct smears and smears from the pus treated with antiformin failed to reveal the presence of acid-fast. Smears from the undiluted pus stained with Loeffler's methylene blue and by Gram's method revealed a few, scattered, gram-positive, short-chained streptococci. Human blood-agar (+1) plates incubated aerobically and anaerobically at 37 C. remained sterile on 2 weeks' incubation. Plates and slants of plain infusion agar (+1) incubated at partial tension at 37 C. yielded within 24 hours a delicate growth of very small streptococcus colonies in pure culture. After 2 successive transplants, 2 days apart, on plain agar (+1) at partial tension, a marked growth was obtained

⁹ Jour. Path. and Bacteriol., 1908, 15, p. 76.

whereas transplants on human blood agar (+1) and plain infusion agar (+1) incubated aerobically at 37 C. remained sterile or yielded only a few minute colonies.

B. Direct smears of the pus and smears made following the antiformin method were negative for acid-fast and other recognizable bacteria. (A later specimen, treated with antiformin, revealed acid-fast. A small quantity of the pus injected intraperitoneally into a 300 gm. guinea-pig caused death in about 5 weeks with general tuberculosis.)

A loopful of undiluted pus was planted into each of 2 tubes of plain infusion broth (+1) and on slants of plain infusion agar (+1). One tube of each medium was incubated aerobically at 37 C. and 1 tube of each series at 37 C. at partial tension. On 3 weeks' continuous incubation, the cultures remained sterile and were discarded.

At the same time the cultures were made, 1 loopful of undiluted pus was evenly distributed on the surface of a slant of plain infusion agar (+1). This culture was placed in a Novy jar along with 3 plain agar (+1) slants inoculated with *B. subtilis*. In the bottom of the jar was placed about 20 cc of a saturated solution of calcium hydroxid, to remove the respiratory carbon dioxide from the system. The jar was sealed with hard paraffin and tightly clamped, and placed in the incubator at 37 C. After 4 days' incubation, the jar was opened. The calcium hydroxid solution was cloudy and possessed a scum of calcium carbonate on the surface. The plain agar culture, when examined by indirect natural light, showed a large number of extremely minute, very translucent, grayish colonies, most numerous on the surface of the slant immediately above the water of syneresis. Microscopically, the colonies were seen to be composed of very minute, very difficultly staining, streptococci (cf. below).

The culture was again sealed in a Novy jar along with 5 freshly inoculated slants of *B. subtilis* and about 20 cc of calcium hydroxid solution and re-incubated at 37 C. At the expiration of 2 days, the colonies showed no increase in size or numbers. Transplants were made to plain infusion agar (+1), Martin's ascitic agar (0.5), Dorset's egg medium, human blood agar (+1) and plain broth. One series of transplants was incubated at 37 C. in a sealed jar along with 4 plain agar (+1) slants of *B. subtilis* and the other series was incubated at 37 C. along with 4 plain agar (+1) slants of *B. subtilis* and about 20 cc of saturated calcium hydroxid solution. All of the tubes remained sterile and after 5 weeks' continuous incubation they were discarded.

MORPHOLOGY AND BIOLOGY

The streptococci isolated from asthma, pyorrhea, of mastoiditis, furunculosis, empyema, stool, urine and the uterus (4), had the following points in common:

In 24-hour plain infusion-agar (+1) and plain infusion-broth (+1) cultures, the cocci appeared as gram-positive, short-chained streptococci, with a considerable number of diplococcal forms and, less frequently, small somewhat irregular clumps which stained readily with ordinary anilin dyes. On human blood agar (+1) and on plain infusion agar at partial tension, the discrete colonies appeared gray and translucent, as did they on these mediums when aerobic growth was obtained. In plain infusion broth (+1) a fine, grayish, granular sediment developed after 24-48 hours' incubation at 37 C. Uniformly, the growth obtained at partial tension on plain infusion agar (+1) and human blood agar (+1) was strikingly greater than that observed on the same mediums when incubated aerobically or anaerobically.

In the remaining cases, the morphology or biology of the streptococci was sufficiently distinctive to merit a special description.

Cold Abscesses.—CASE 1.—This streptococcus, isolation of which was rendered possible only by the partial tension method, appeared as a small, gram-positive nonacid fast, short-chained streptococcus, the single cocci averaging about 0.4-0.5 mikrons in diameter. Staining was easily effected with the ordinary anilin dyes. The adaptation of this strain to growth at partial tension is suggested by the fact that its isolation was effected only by incubation of the cultures under diminished oxygen tension, as well as by the fact that subsequent partial tension transplants yielded a moderately heavy growth, whereas aerobic transplants yielded, at best, only a few, tiny scattered colonies and, in some cases, no growth at all. Even at partial tension, however, the primary cultures were characterized by their relative delicacy of growth.

CASE 2.—The cultural requirements of the strain isolated from this case were unique, in that isolation was effected only by incubating the culture under conditions of partial oxygen tension plus the removal of the respiratory carbon dioxid. As will be recollected, the growth on plain agar under such conditions appeared as very minute, very translucent colonies, even difficultly visible when examined by indirect natural light. Growth appeared within 4 days' incubation at 37 C. Transplants failed to develop.

Smears from single colonies, when stained with Loeffler's methylene blue for 2 minutes, Gram's method, warm, dilute (1:20) carbol-fuchsin for several hours, and by the Ziehl-Neelsen carbolfuchsin method, revealed considerable numbers of very minute, very feebly staining coccoid bodies, which took the stain so lightly that they were not visible when examined by strong, artificial illumination. When stained for 5 minutes with a saturated alcoholic solution of victoria blue (Grubler), the micro-organisms stained rather readily and appeared under the ordinary Leitz 1:12 oil immersion lens and No. 3 ocular as very minute cocci of rather constant size, and so small as to be but little above the limit of visibility. These bodies occurred as diplococci and in short chains of from 4-5 members. Comparison with a smear from a culture of the globoid bodies of poliomyelitis, obtained through the kindness of Dr. Noguchi, revealed that the cocci were of about the size of the minute forms of the globoid bodies described by Flexner and Noguchi¹⁰ and Rosenow.¹¹

¹⁰ Jour. Exper. Med., 1913, 18, p. 461.

¹¹ Jour. Infect. Dis., 1918, 22, p. 281.

Uterus (Chorea).—In this case, which was one of very severe chorea, an obligative partial tension streptococcus was isolated in pure culture from the uterus a few hours prior to death. The microbe was a minute, gram-positive, short-chained streptococcus, very slightly larger than the second strain isolated from a cold abscess. It was isolated on plates of Martin's ascitic agar (0.5) incubated at partial tension. Aerobic and anaerobic cultures remained sterile. The colonies were minute, gray and translucent and in the primary partial tension culture numbered only six to eight. Transplants to human blood agar (+ 1), made partial tension, yielded a more marked growth, whereas aerobic transplants to the same medium remained sterile. Recently, Quigley¹² has reported a series of 21 cases of chorea in which he isolated a delicately-growing short-chained streptococcus from both the blood and spinal fluid, and in 14 cases from either one or the other.

Puerperal Sepsis.—In these 3 cases, which followed in the wake of the widespread respiratory infections which swept New York City during the winter of 1917-1918, rather similar streptococci were isolated in pure culture from the uterus and in mixed culture from the throat. The organisms occurred as gram-positive, long-chained streptococci. In 24 hour plain and dextrose infusion-broth (+ 1) cultures, chains of 6-8 cocci occurred, but the predominant forms were long chains of from 12-30 or more members, a diplococcus grouping being often rather prominent in the chains.

In plain infusion broth, after 24-48 hours' incubation at 37 C., a gray flocculent growth was visible at the bottom and on the side of the test tube. The supernatant broth remained unclouded. All of the strains were bile-insoluble and failed to ferment inulin aerobically or at partial tension.

The uterine strains, on isolation, exhibited a more marked adaptation to growth at partial tension than did the strains isolated from the throat. The former, moreover, grew better anaerobically than aerobically, whereas the reverse was true in the case of the throat strains. However, all exhibited their most marked growth under conditions of diminished oxygen tension.

Blood cultures on these cases at various stages of the uterine infection remained sterile. The plates were incubated at 37 C. both aerobically and at partial tension.

¹² Jour. Infect. Dis., 1918, 22, p. 198.

VACCINES

In 17 of the 32 cases reported, vaccines were prepared from the growth obtained at partial tension. In none of the cases was sufficient growth obtained aerobically within 48 hours' incubation at 37 C. to allow of the preparation of a vaccine, whereas in all cases sufficient growth resulted at partial tension within 2 days and, in certain cases, within 24 hours, thus allowing of the preparation of an autogenous vaccine without delay. In certain of the cases, the factor of time in the preparation of the vaccine was of importance.

In 3 cases of pyorrhea and 3 cases of asthma the reaction and the clinical improvement in the patient was rather marked following the administration of vaccine. In each case, a 24-48 hour growth on plain agar (+ 1) or dextrose agar (+ 1) was employed. The initial dosage was 1 minim of vaccine (equivalent to 5-10 million streptococci), the injections being made subcutaneously at 3-day intervals. The dosage was gradually increased up to 12-15 minims.

Although it was not feasible to treat a parallel series of cases with a vaccine prepared from growth obtained aerobically, and although it is often extremely difficult, to say the least, to determine exactly what share in the clinical improvement of a patient is attributable to the vaccine, yet it is at least theoretically possible that the antigen obtained by cultivating certain micro-organisms at partial tension may be modified or different from the antigen obtained by growth aerobically.

SUMMARY

From a series of 32 cases of rather diverse nature partial tension streptococci were isolated. In 1 case of asthma, 1 of pyorrhea, 1 of chorea, 1 of uterine infection and 2 of "cold abscess" growth was obtained only at partial tension. In one of the cases of "cold abscess" isolation of a very minute streptococcus was effected only by growth in an atmosphere of diminished oxygen from which the respiratory carbon dioxid was removed. In the remaining 26 cases, the relative luxuriance of growth at partial tension was in contrast to the meager growth obtained aerobically and, in certain cases, anaerobically.

By the use of the partial tension method sufficient growth was obtained usually within 24 hours and never later than 48 hours, to allow of the preparation of an autogenous vaccine. In none of the 17 cases in which vaccines were prepared was sufficient growth obtained aerobically or anaerobically within 48 hours to warrant the preparation of a vaccine.